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Rapid and sensitive analytical method for the determination of amoxicillin and related compounds in water meeting the requirements of the European union watch list

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ABSTRACT

The presence of antibiotics in the aquatic environment is becoming one of the main research focus of scientists and policy makers. Proof of that is the inclusion of four antibiotics, amongst which is amoxicillin, in the EU Watch List (WL) (Decision 2020/1161/EU)) of substances for water monitoring. The accurate quantification of amoxicillin in water at the sub-ppb levels required by the WL is troublesome due to its physicochemical properties. In this work, the analytical challenges related to the determination of amoxicillin, and six related penicillins (ampicillin, cloxacillin, dicloxacillin, penicillin G, penicillin V and oxacillin), have been carefully addressed, including sample treatment, sample stability, chromatographic analysis and mass spectrometric detection by triple quadrupole. Given the low recoveries obtained using different solid-phase extraction cartridges, we applied the direct injection of water samples using a reversed-phase chromatographic column that allowed working with 100% aqueous mobile phase. Matrix effects were evaluated and corrected using the isotopically labelled internal standard or correction factors based on signal suppression observed in the analysis of spiked samples.

The methodology developed was satisfactorily validated at 50 and 500 ng L^{-1} for the seven penicillins studied, and it was applied to different types of water matrices, revealing the presence of ampicillin in one surface water sample and cloxacillin in three effluent wastewater samples.

1. Introduction

According to the most recent report from the European Centre for Disease Prevention and Control [1], the average consumption of antimicrobial drugs for systemic use in the European Union in 2019 was 18.0 defined as daily doses per 1000 inhabitants per day. After consumption, antibiotics are excreted as metabolites and/or unaltered compounds [2,3], and together with the high consumption of these compounds, it is not surprising that these compounds reach the aquatic environment through wastewater treatment plant (WWTP) discharges [4]. Thus, the investigation of antibiotic residues in water has become an important topic in environmental science, including the analytical determination at trace levels (ng L^{-1} or µg L^{-1}) [5–8]. Antibiotics are of special concern due to their potential to produce bacterial resistance [2,9,10]. In order to obtain an accurate picture of their occurrence and potential harmful effects in the aquatic environment, highly sensitive analytical methods that allow the detection, identification and quantification of antibiotics in environmental matrices are required [11–13].

The European Watch List (WL) of substances for Union-wide monitoring in the field of water policy has recently included four antibiotics, namely ciprofloxacin, sulfamethoxazole, trimethoprim, and amoxicillin [14]. The substances on this WL are selected because the information available indicates that they may pose a significant risk to or via the aquatic environment, but monitoring data is still insufficient in order to reach a conclusion on their actual risk. While ciprofloxacin, sulfamethoxazole, trimethoprim are frequently included in multi-class methods for pharmaceuticals [15–17], the highly polar amoxicillin normally requires different procedural and measurement conditions for its determination at low sub-ppb concentration levels. Amoxicillin (**Figure S1, Supplementary Information**) is a β-lactam antibiotic drug belonging to penicillins and it is considered as an essential medicine by the World Health Organisation due to its pharmacological properties capable to treat pneumonia, pharyngitis, sepsis, sinusitis or bacterial meningitis [18]. The WL establishes a maximum acceptable detec-

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tion limit for amoxicillin of 78 ng L^{-1} in surface water samples, and recommends its determination by solid-phase extraction (SPE) followed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis [14]. Although several studies have reported the determination of amoxicillin by SPE-LC-MS/MS in wastewater [5,19,20] and surface water [11,13,14,15], its high polarity implies difficulties to achieve its optimal extraction, leading to recoveries commonly below 70% [4,12], and even requiring the use of HILIC columns for obtaining appropriate chromatographic retention [5].

The importance of amoxicillin, highlighted by its inclusion in two consecutive EU Watch lists (2018 and 2020), and the difficulties associated to its determination, justify the development of updated analytical methodologies that meet the requirements of the EU. In this study, specific analytical methodology has been developed for the rapid and robust determination of amoxicillin in surface water, together with another six related penicillin antibiotics (ampicillin, cloxacillin, dicloxacillin, penicillin G, penicillin V and oxacillin). Two methodologies, one based on SPE and another on the direct injection (DI), were studied using an ultra-high performance liquid chromatography (UHPLC)-MS/MS system with triple quadrupole mass analyser. The methodology was optimised in order to maximize sensitivity and chromatographic performance, and it was finally applied to surface water samples as well as to other type of sample matrices, such as effluent wastewater and groundwater. Additionally, a stability study of these compounds in surface water was performed to evaluate sample storage and analyte stability.

2. Materials and methods

2.1. Reagents and chemicals

Seven penicillin antibiotics (**Figure S1, Supplementary Information**) were selected (amoxicillin, ampicillin, cloxacillin, dicloxacillin, penicillin G, penicillin V and oxacillin), whose analytical standards were purchased from LGC Standards (UK). Due to their presumable degradation in methanol solutions [21], stock standards were prepared in water:acetonitrile 25:75 (v:v). A mix solution containing the seven antibiotics selected was prepared at a concentration level of 5 mg L^{-1}. Working mixed solution and calibration curves were daily obtained by dilution of the stock mix with Milli-Q water. Amoxicillin-$^{13}C_6$ (isotopically-labelled internal standard, ILIS) was purchased from LGC Standards.

LC-MS grade water was obtained by purifying demineralized water using a Milli-Q system from Millipore (Bedford, MA, USA). Methanol and acetonitrile (LC-MS grade), formic acid (>98%), ammonium acetate (>98%) and ammonium fluoride (>98%) were purchased from Scharlau (Scharlab, Barcelona, Spain).

Different SPE cartridges purchased from Waters (Milford, USA) were tested: Oasis HLB (60 mg and 150 mg), Oasis MCX (150 mg) and Oasis MAX (150 mg). Syringe filters (13 mm and 0.22 µm) tested were made of nylon, PTFE hydrophobic and PTFE hydrophilic.

2.2. Water samples

For the optimization of the analytical methodology, five surface water samples were collected from different sites of the Castellon province (Spain): SW1 (*Séquia de l'Obra* irrigation ditch; 40.0199, 0.0034; Castelló de la Plana), SW2 (*Clot de la Mare de Déu*; 39.8710, −0.0595; Burriana), SW3 (*Estany de Nules*; 39.8330, −0.1066; Nules), SW4 (*Estany d'Almenara*; 39.7516, −0.1942; Almenara), and SW5 (*Millars* River; 39.9539, −0.0979; Almazora). In all cases, sampling points were selected trying to avoid possible presence of pharmaceuticals, as they were far enough of any potential source of these compounds.

The analytical methodology was finally applied to fourteen different type of water samples: 3 surface waters collected in different sites of the *Millars* River, 7 ground water samples from wells sited in the surrounding of *Millars* River, and 4 effluent wastewater samples (24-h composite) collected at different days from the WWTP of the city of Castelló.

All samples were collected in 500 mL amber glass bottles, transported in refrigerated isothermal containers keeping on ice and stored at −23 °C until analysis (max. 30 days).

2.3. Sample treatment

The excellent sensitivity of modern LC-MS/MS instrumentation allowed direct injection (DI) of samples without any preconcentration step, similarly to other methods developed for pharmaceuticals [8,22–24]. After centrifuging of 2 mL sample at 12,000 rpm for 15 min, 950 µL of supernatant was transferred into a glass vial and 50 µL of the ILIS solution of 2 $\mu g\ L^{-1}$ (100 ng L^{-1} in vial) was added. Finally, 100 µL of the final solution were injected into the LC-MS/M system.

2.4. Stability study

A stability study was performed in two surface water samples (SW3 and SW5, randomly selected). Samples were spiked at 800 ng L^{-1} and stored at 4 °C and −23 °C. Analysis were performed at different time intervals (0, 3, 7, 14, 21 and 30 days, for samples stored at 4 °C; 30 days, for samples stored at −23 °C).

2.5. Instrumentation

An Acquity UPLC H–Class liquid chromatography system (Waters Corp, Milford, MA, USA) interfaced to a Xevo TQ-S triple quadrupole mass spectrometer (Waters Corp, Manchester, UK) equipped with a Z-Spray electrospray (Waters Corp, Manchester, UK) was used for sample analysis. For chromatographic separation, an Atlantis T3 3.0 × 150 mm, 3 µm analytical column (Waters Corp, Wexford, Ireland) maintained at 40 °C was used. Mobile phases consisted on water (solvent A) and methanol (solvent B), both with 1 mM ammonium fluoride, delivered at a flow rate of 0.4 mL min^{-1} and changing as follows: 0 min 0% B, 0.5 min 0% B, 6.0 min 99% B, 8.0 min 99% B, and 8.1 min 0% B maintained to 10 min for column re-equilibration. Injection volume was 100 µL.

ESI was operated in positive ionization mode (ESI^+) using a capillary voltage of 1.0 kV. Nitrogen was used as desolvation (1200 L h^{-1}) and cone gas (250 L h^{-1}), while source temperature was set to 150 °C, and desolvation temperature 650 °C. Cone voltage and collision energies, using argon (99.995%, Praxair) as collision gas, were optimized for each compound. Three selected reaction monitoring (SRM) transitions were acquired per compound (quantification transition *Q*, first confirmation transition q_1, second confirmation transition q_2). Dwell times were automatically selected in order to acquire 12 points/peak, being at least 14 ms/transition.

UHPLC-MS/MS data were acquired and processed using MassLynx 4.1 software (Waters Corp, Manchester, UK) and TargetLynx application (Waters Corp, Manchester, UK).

2.6. Validation

The analytical methodology was validated evaluating the following parameters:

Specificity was tested by the analysis of the five surface samples previously described, without detecting chromatographic peaks fitting the selected SRM transitions.

Linearity was evaluated by analysing calibration curves prepared in Milli-Q water at 7 concentration levels: 10, 25, 50, 100, 250, 500, 1000 ng L^{-1}. Linearity was assumed when regression coefficient was >0.99 with residuals lower than 20%.

Accuracy was evaluated by the analysis of five surface water samples spiked at 50 ng L^{-1} and 500 ng L^{-1} in triplicate. As the method was based on direct injection, the observed recoveries indicated the matrix effect in the surface water samples used for method validation; therefore, the use of amoxicillin-$^{13}C_6$ as ILIS allowed to evaluate the correction of matrix effects.

Precision was evaluated in the five spiked surface waters at each fortification level assayed ($n = 3$) in terms of relative standard deviations (RSD). Values lower than 20% were considered as satisfactory.

Limit of quantification (LOQ) was the lowest validated level with acceptable recovery and precision (i.e., 50 ng L^{-1}).

Limit of detection (LOD) was established from the spiked surface water sample at 50 ng L^{-1} that presented the highest matrix effect (i.e. the worst case scenario) for a signal-to-noise ratio (S/N) of 3, calculated for the less sensitive qualitative transition (q_2), as the 3 SRM transitions should be observed for compound identification.

3. Results and discussion

The focus of this study was the development of analytical methodology for the determination of amoxicillin in surface water samples, as it is currently included in the WL of substances for European-wide monitoring in the field of water policy [14] with a maximum acceptable method detection limit of 78 ng L^{-1}. Thus, the instrumental conditions, chromatographic separation and sample treatment were optimized for this compound. Subsequently, the same conditions were applied for the rest of penicillins selected, in order to develop a multi-residue method for the determination of the seven antibiotics included in this work.

3.1. Mass spectrometry considerations

Amoxicillin could be measured only in ESI^+, as no ionisation was observed in ESI^- in spite of the presence of the carboxylic acid moiety. This antibiotic presents a primary amine moiety which is expected to be the protonation site in ESI^+, based on the observed MS spectrum and product ions. **Fig. 1A** shows the MS scan of amoxicillin, with the base peak at *m/z* 349, despite that the protonated molecule has an *m/z* of 366. The *m/z* 349 ion resulted from an ammonia neutral loss (17 Da), which suggested that the protonation site of amoxicillin was at the primary amine moiety. The in-source fragmentation of primary amine or terminal amide moieties producing ammonia loss has been reported in literature, for example for amphetamine [25] or phenethylamine with a terminal amide [26]. However, this phenomenon was not observed in our study for ampicillin even though its structure is similar to amoxicillin. Three SRM transitions were optimized for each precursor ion, *m/z* 349 (amoxicillin-NH_3) and *m/z* 366 (amoxicillin) in order to compare sensitivity and selectivity. The selection of the amoxicillin in-source fragment *m/z* 349 as precursor ion increased the sensitivity in the three selected transitions **(Fig. 1C**) when compared to the protonated molecule (**Fig. 1B**). Additionally, the transition 349>208 (amoxicillin-NH_3) presented higher selectivity than SRM 366>208 (amoxicillin), allowing to reach lower LODs. Therefore, up to six transitions could be used for the determination of amoxicillin, even combining those from different precursor ions. Finally, only those transitions resulting from precursor-ion *m/z* 349 (amoxicillin-NH_3) were selected to minimize the effect of potential variations of the in-source fragmentation due to the matrix, and to obtain robust ion intensity (*q*/*Q*) ratios for compound identification, as selecting different precursor ions could lead to higher variations in *q*/*Q* ratios.

After optimization of MS/MS conditions for amoxicillin, the acquisition parameters in ESI^+ were also determined for the rest of the antibiotics selected (see **Table 1**).

3.2. Chromatographic separation

Different organic solvents (methanol and acetonitrile) with combinations of commonly used modifiers (formic acid, ammonium acetate and ammonium fluoride) were tested in order to maximize sensitivity and improve peak shape for amoxicillin. Instrumental sensitivity was crucial to reach the detection limit (78 ng L^{-1}) required for amoxicillin in the European WL [14] and because of the absence of pre-concentration steps. The most satisfactory results were obtained using water and methanol, both with 1 mM ammonium fluoride, being the mobile phase selected for further experiments. The use of ammonium fluoride in ESI^+ for signal enhancement and peak shape improvement has been reported in literature [27,28], showing in some cases a better performance than ammonium acetate, in accordance to what we observed in our study.

Different chromatographic columns have been reported for amoxicillin determination, such as C18 [13,19,20,29–31], phenyl-based [12,32,33], monolithic [34], or HILIC [5]. In this study, two columns were tested, a Cortecs C18 (2.7 µm, 2.1 × 100 mm) and an Atlantis T3 (3 µm, 3.0 × 150 mm). A comparison of results obtained both columns are shown in **Fig. 2**. The only difference in the chromatographic gradient applied was in min 0, being for Cortecs 5% B and for Atlantis 0% B. The CortecsC18 column produced a 30 s-width poorly-defined peak, while the Atlantis T3 led to a 9 s-width peak almost symmetric. The poor retention in C18 could be explained by the high polarity of amoxicillin, as well as the fact that this compound is a zwitterion at the working pH in UHPLC according to its pKa values reported in PubChem (https://pubchem.ncbi.nlm.nih.gov/compound/33613). Poor retention of amoxicillin using C18 column has been also reported by Rossmann et al. [5] who proposed HILIC separation. Reversed-phase analytical columns that allow the use of 100% aqueous mobile phases, such as the Atlantis T3, are highly recommended for retention of polar compounds, similarly to HILIC separations but without using highly-concentrated buffers. Additionally, the use of reversed-phase columns allows the direct injection of aqueous samples, while for HILIC separations the sample extract uses to be dissolved in acetonitrile. Therefore, the Atlantis T3 column was selected for further optimization.

The chromatographic conditions optimized for amoxicillin were also suitable for the rest of antibiotics selected, with appropriate retention times and peak shapes (**Table 1**).

3.3. Sample treatment

3.3.1. Solid phase extraction

As the recommended analytical procedure for amoxicillin determination by the WL is SPE-LC-MS/MS [14], we tested several cartridges and conditions (Oasis HLB, Oasis MCX, Oasis MAX, Strata-X, and acidified sample for Oasis HLB and Strata-X, both with 1% formic acid). Some of these cartridges have been previously reported for amoxicillin determination, (e.g. Oasis HLB, Strata-X [5,10,12,13,20]; Oasis MCX [35]).

The first experiments were performed with Milli-Q water fortified at 50 ng L^{-1} (**Fig. 3**). Unlike the rest of antibiotics studied, not enough retention was observed for amoxicillin in the cartridges tested, which is in accordance with its polar nature. These results are in accordance with other works showing low recoveries for amoxicillin (30–75%) using polymeric cartridges, such as Oasis

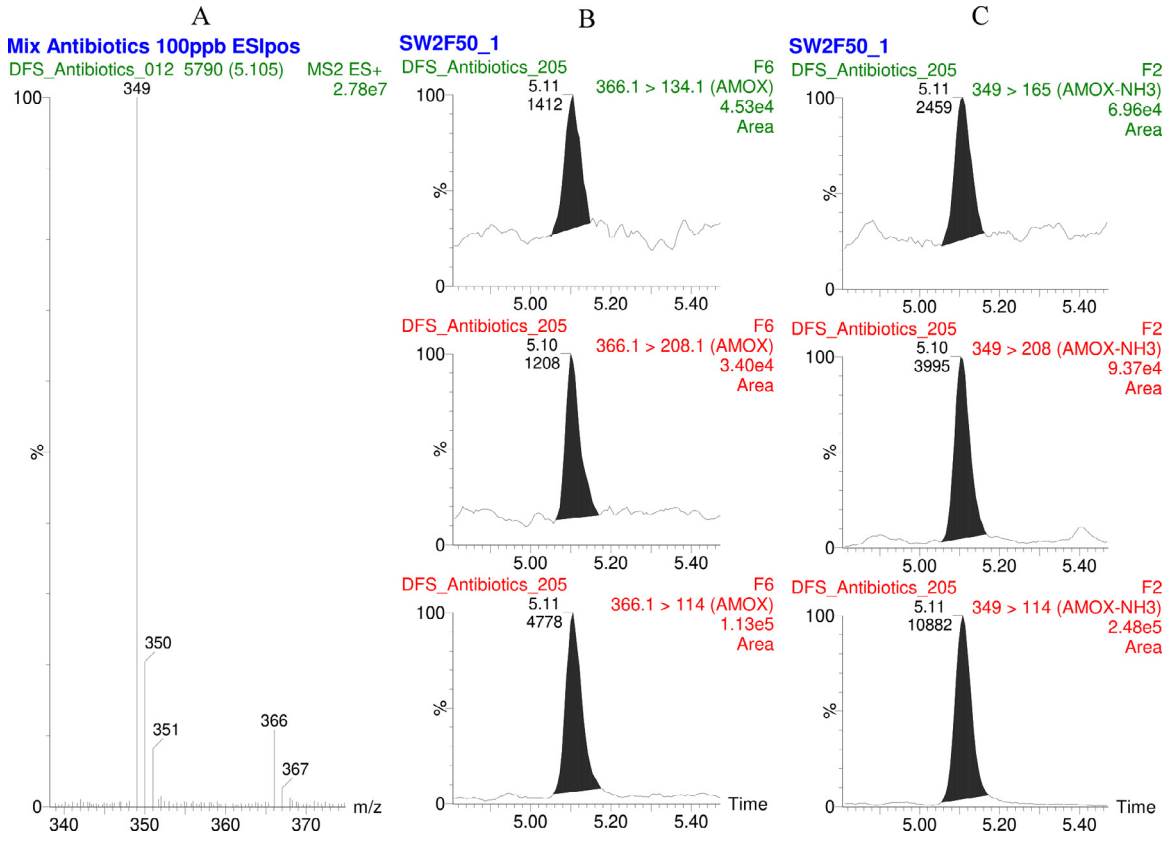

Fig. 1. Mass spectrometric optimisation of amoxicillin. (**A**) Scan spectrum of amoxicillin standard at 100 µg L^{-1} showing the protonated molecule (*m/z* 366) and the in-source fragment resulting after ammonia loss (*m/z* 349). SRM chromatograms for amoxicillin (**B**) and amoxicillin-NH_3 (**C**).

Table 1
UHPLC-MS/MS acquisition parameters for the antibiotics selected (quantification (Q) and confirmation (q_1 and q_2) transitions).

Compound	RT (min)	Precursor ion	CV (V)	Q transition		q_1 transition		q_2 transition	
				Product ion	CE (eV)	Product ion	CE (eV)	Product ion	CE (eV)
Amoxicillin-NH_3	5.13	349.0	40	114.0	15	208.0	10	165.0	20
Ampicillin	6.34	350.1	20	106.0	20	160.0	10	192.1	15
Cloxacillin	7.57	436.1	20	160.0	15	277.0	15	178.0	30
Dicloxacillin	7.76	470.0	20	160.0	10	310.9	15	212.1	35
Penicillin G	7.20	335.1	20	160.0	10	176.1	10	114.0	30
Penicillin V	7.44	351.1	20	160.1	10	114.0	30	86.9	35
Oxacillin	7.47	402.1	20	160.1	15	243.1	10	144.0	30
Amoxicillin-$^{13}C_6$–NH_3	5.13	355.0	40	114.0	15				

RT: retention time.
CV: cone voltage.
CE: collision energy.

HLB or Strata-X [4,5,12], which may compromise the use of SPE for pre-concentration of water samples. In addition, SPE procedure for these antibiotics are pH-dependant [4], and acidic conditions can cause compound degradation [21,36]. The zwitterionic character of amoxicillin adds an extra handicap for its determination, as the pH of the sample should be carefully adjusted in order to maximize compound retention, but keeping in mind the degradation of beta-lactams under acidic conditions.

On-line SPE systems, using C18 [4,21] or monolith columns [34], have been also proposed for the determination of beta-lactam antibiotics, but this configuration was not tested in our work as it was not available at our laboratory. Considering the low recoveries obtained for amoxicillin in all cartridges tested and the data reported in the literature, the use of SPE as sample treatment was discarded and the DI of water samples was assayed.

3.3.2. Direct injection

Procedures based on DI of the sample have been successfully applied in our laboratory for the determination of pharmaceuticals in aquatic samples [8,22–24]. In this work, we tested the feasibility of this simple and rapid approach, which requires the use of modern LC-MS/MS instrumentation, for the determination of penicillins selected at trace levels.

Previously, the application of a sample filtering step was studied in order to evaluate possible analyte losses. Three different syringe filters (nylon, hydrophobic PTFE and hydrophilic PTFE, all of them of 13 mm and 0.22 µm) were tested with Milli-Q water spiked with penicillins at 100 ng L^{-1}. Fig. 4 shows the results obtained for the three filters tested compared with no filtering step. It can be seen that all antibiotics experienced losses when filtering the sample, especially using nylon. This fact illustrates the impor-

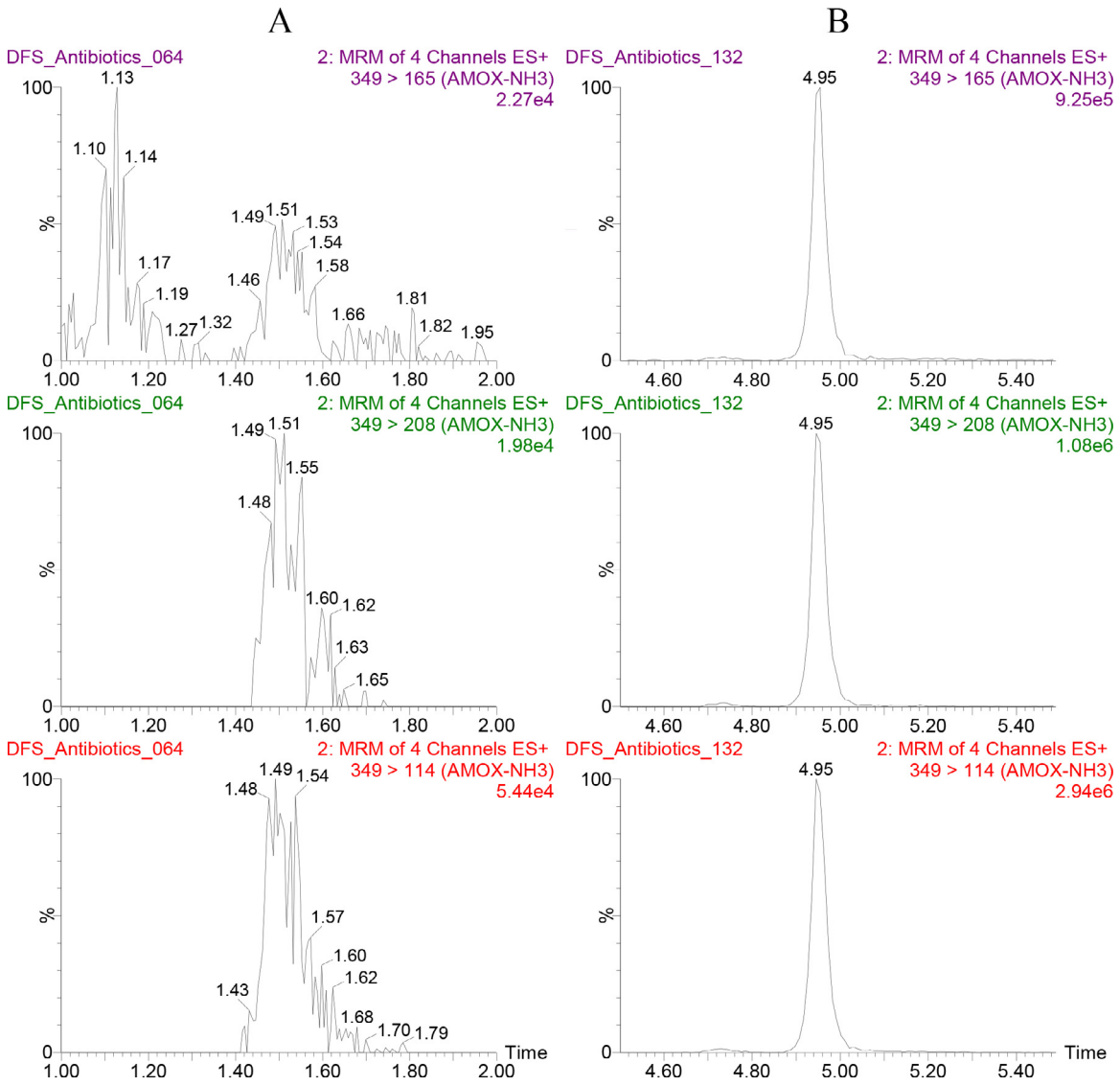

Fig. 2. Chromatographic optimisation of amoxicillin. (**A**) Chromatograms obtained with Cortecs C18 column. (**B**) Chromatograms obtained with Atlantis T3 column.

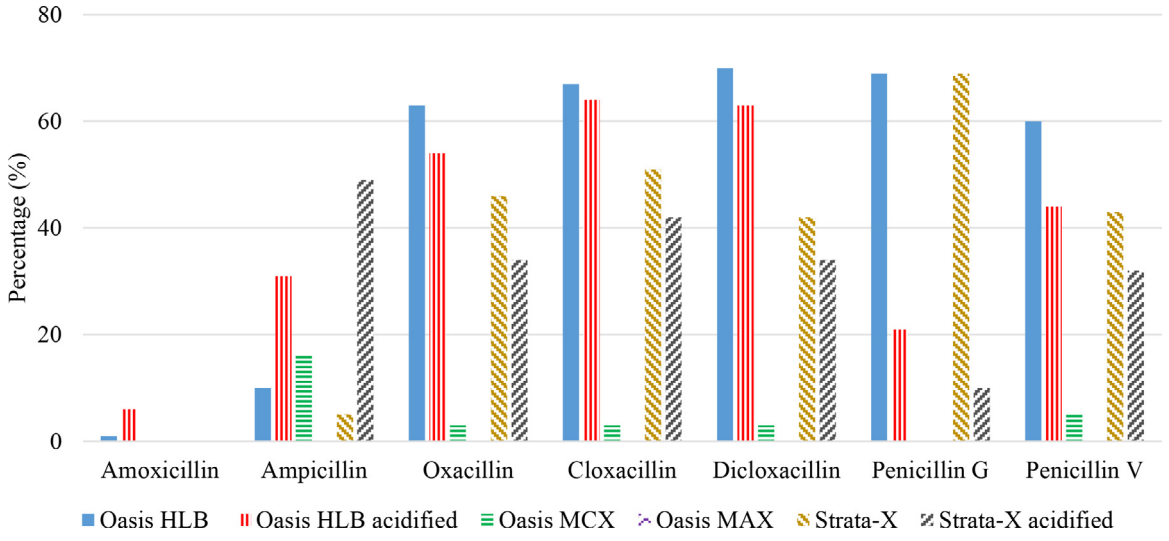

Fig. 3. Recoveries (%) obtained for the studied antibiotics using different SPE cartridges.

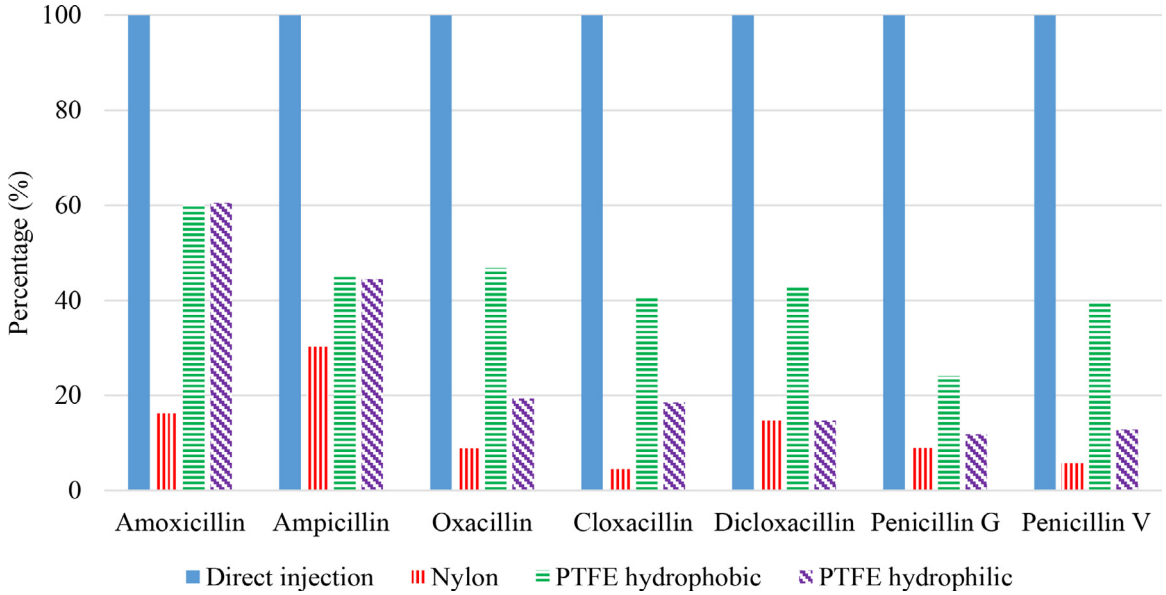

Fig. 4. Antibiotic recoveries (%) after using different syringe filters before the DI of Milli-Q water spiked at 100 ng L^{-1}.

tance of evaluating the filtering step when developing analytical procedures for organic micro-pollutants. Once filtering was evaluated and discarded for this study, samples were centrifuged at 12,000 rpm during 15 min prior to analysis. A sample volume injection of 100 µL was selected for UHPLC-MS/MS analysis. This volume of sample injected did not suppose a chromatographic performance problem using an Atlantis T3 analytical column (100% aqueous mobile phase during the first 0.5 min).

It might be expected that the direct injection of 100 µL of surface water would lead to more severe matrix effects. Therefore, different water samples spiked at 50 ng L^{-1} were analysed using the described procedure in order to evaluate matrix effects. A signal suppression up to 50% was observed in some cases, together with a 0.14 min chromatographic retention time shift (exceeding the ±0.1 min tolerance established in some guidelines [37]), as it can be observed in **Figure S2** for amoxicillin in the five selected surface samples used for validation. Hence, the use of ILIS for correcting these variations is highly recommendable to compensate for the effects of the matrix sample on the determination of penicillins in water.

3.4. Method validation

Keeping in mind the development of an analytical method for amoxicillin at concentrations below the detection limit established by the WL (78 ng L^{-1}), we used Amoxicillin-$^{13}C_6$ as ILIS for correction of matrix effects, and we also tested its applicability for the remaining analytes. Two SRM transitions were acquired for the ILIS, in order to test the best option for matrix effects correction: 1) amoxicillin-$^{13}C_6$–NH_3, i.e. using the ion-source fragment as precursor ion similar to amoxicillin, and 2) as protonated molecule like for the rest of the penicillins.

In absence of universal guidelines widely accepted in environmental analytical chemistry, we followed the spirit of SANTE, a strict guideline applied to pesticide residue analysis in food, biological and water samples [37]. Linearity (from 10 to 1000 ng L^{-1}) was satisfactory for all compounds, with the residuals below 20% and correlation coefficients higher than 0.99. Precision and accuracy were evaluated by analysis of five different surface waters spiked at 50 and 500 ng L^{-1} with the seven antibiotics investigated, in triplicate**.** Non-spiked surface water samples were also analysed in order to ensure selectivity of the method, revealed by the absence of chromatographic peaks for the selected SRM. **Table 2** shows the summary of the validation results, where a satisfactory precision (RSD $\leq$ 10%) can be seen for all compounds. As the method was based on direct injection, the recoveries actually indicated the matrix effect for the compounds in each surface water sample. The signal suppression of amoxicillin was the only one that could be satisfactorily corrected in all samples by using its own ILIS, obtaining recoveries between 102 and 110%. For the rest of penicillins, the potential correction with amoxicillin-$^{13}C_6$ as ILIS did not improve the results obtained, so their recoveries were calculated without using ILIS. Despite the absence of ILIS for the remaining compounds, their recoveries were rather satisfactory (mostly between 60 and 90%), although important signal suppressions were observed in the sample SW3, for which recoveries around 40% were obtained for some analytes.

It is well known that matrix effect depends on both the analyte characteristics and the matrix composition. In a rapid and direct method, as the one developed in this paper, the absence of the own analyte-ILIS makes the correction of matrix effects complicated. Consequently, while amoxicillin was satisfactorily quantified by using its own ILIS, a correction factor (Cf) based on the signal suppression observed should be applied for the remaining penicillins. The analytical strategy proposed for the rest of antibiotics consists on the analysis of the water sample in duplicate, analysing the non-spiked sample and also a quality control (QC), i.e. the sample spiked with the antibiotics at 100–200 ng L^{-1} for signal suppression evaluation and estimation of the Cf for each sample. Then, the application of Cf to the non-spiked sample already analysed would allow its accurate quantification. The Cf is estimated from the QC recovery obtained (e.g. a QC recovery of 60% gives Cf=1.67, which means that the analyte concentration in this sample should be multiplied by 1.67 to provide more reliable data). This strategy is practical and little time-consuming because the method does not include any sample treatment (only a centrifugation step). As the own analyte-ILIS is used for amoxicillin, it is not necessary to apply any correction factor for its determination due to the efficient matrix effects correction of the ILIS.

The LOQ was established as the lowest level validated, i.e. 50 ng L^{-1}, for all the compounds [37], although lower concentrations could have been tested lowering the LOQ if needed. In this way,

Table 2
Recoveries (%) (RSD in brackets) obtained for the validation of antibiotics selected in surface water samples spiked at two concentration levels in triplicate. Limit of detection (LOD) is also shown.

Compound	LOD (ng L^{-1})	*SW1*		*SW2*		*SW3*		*SW4*		*SW5*	
		50 ng L^{-1}	500 ng L^{-1}	50 ng L^{-1}	500 ng L^{-1}	50 ng L^{-1}	500 ng L^{-1}	50 ng L^{-1}	500 ng L^{-1}	50 ng L^{-1}	500 ng L^{-1}
Amoxicillin[1]	9	105 (1)	105 (1)	105 (1)	105 (4)	105 (5)	110 (1)	102 (3)	107 (2)	104 (3)	106 (3)
Ampicillin[2]	0.5	96 (1)	85 (1)	88 (2)	78 (4)	75 (1)	63 (2)	82 (6)	75 (10)	83 (5)	73 (10)
Cloxacillin[2]	0.7	68 (3)	75 (3)	67 (2)	69 (5)	51 (2)	60 (4)	78 (3)	84 (5)	76 (1)	81 (3)
Dicloxacillin[2]	2	62 (5)	80 (3)	61 (2)	72 (5)	48 (6)	65 (4)	71 (3)	89 (6)	74 (3)	90 (2)
Penicillin G[2]	0.2	68 (2)	77 (1)	63 (2)	72 (4)	48 (0)	59 (2)	73 (2)	86 (6)	73 (2)	82 (2)
Penicillin V[2]	7	61 (3)	70 (6)	55 (6)	64 (6)	38 (5)	49 (3)	69 (7)	82 (8)	77 (3)	84 (3)
Oxacillin[2]	0.6	60 (1)	72 (7)	56 (3)	68 (2)	42 (3)	56 (3)	68 (2)	82 (4)	70 (0)	79 (3)

[1] Determined as amoxicillin-NH_3 and corrected with amoxicillin-$^{13}C_6$ used as ILIS.
[2] Without correction with ILIS.

amoxicillin can be quantified in surface water at concentration levels below the limit of detection established by the WL (78 ng L^{-1}). The excellent method sensitivity allowed to reach very low LODs, ranging from 0.2 to 9 ng L^{-1} (**Table 2**), with reliable identification at these levels thanks to the acquisition of several MS/MS transitions. As an example, **Fig. 5** shows the chromatograms (*Q* transition) for the seven penicillins under study in sample SW5 spiked at 50 ng L^{-1}.

3.5. Antibiotic stability in surface water

Firstly, stability of amoxicillin in surface water samples was tested in order to evaluate its possible degradation during samples storage. To this aim, two different surface water samples (SW3 and SW5) were spiked with amoxicillin at 800 ng L^{-1} and stored at two temperatures, 4 and −23 °C. Analyses were performed at different storage times as indicated in Section 2.4. The measured compound concentration was normalized to the concentration obtained at the time 0 for graphical representation.

Fig. 6 shows that amoxicillin was stable for at least 7 days at 4 °C, starting a moderate degradation after 14 days in the two matrices tested. After 30 days at 4 °C, amoxicillin suffered notable degradation in surface water (32% and 47%). Analysis of the samples after 30 days at −23 °C demonstrated that amoxicillin was stable under these conditions of storage.

In other studies, no significant decline of amoxicillin was observed in methanol-water (50:50) for 12 days at −20 °C [38], or in deionised water at 4 °C after one month of storage [39]. Cha and colleagues found no significant degradation of β-lactams over a storage in acetonitrile-ethanol-water (25:25:50) for 10 days at −20 °C [36]. On the contrary, Borrull et al. found a progressive decrease in the responses of amoxicillin QCs prepared in surface and drinking water even during their storage in the autosampler [40]. The poor stability of amoxicillin in environmental water might be explained by the limited stability of the β-lactam ring [40].

According to the data obtained in this work, surface water samples can be stored either at 4 °C for 7 days, or at −23 °C for 30 days, without significant losses of amoxicillin (degradation below 10%). The stability study was extended to the rest of penicillins selected obtaining similar results (**Figure S3**).

3.6. Application to different types of water samples

The validated methodology was applied to the analysis of 14 samples of different types (see Section 2.2). The objective was to support the applicability of the method to different water matrices, to test the matrix effects and their efficient correction, and to evaluate the accuracy through the recoveries obtained for the QC samples analysed. In the case of effluent samples, a 2-fold dilution was applied before direct injection analysis following a procedure previously developed in our laboratory for pharmaceuticals in wastewater [24]. As the method was not validated for groundwater and effluent wastewater, these samples were analysed by triplicate: without spiking, and after spiking at 50 and 500 ng L^{-1} (i.e. the spiked samples served serving as quality controls). In the case of groundwater, signal suppression was similar to surface water, while for effluent wastewater, suppression between 50 and 85% was observed depending on the compound.

The identity of the antibiotics found in the samples was confirmed by their chromatographic retention time and at least one *q*/*Q* ratio with a deviation lower than 30% in relation to the reference standard. Ampicillin was the only compound that could be quantified (116 ng L^{-1}) in one *Millars* River surface water samples (collection site 39.9229, −0.0411, 1500 m downstream of the Almazora WWTP discharge point) (see **Figure S4**). In the case of groundwater, collected from different points near the *Millars* River

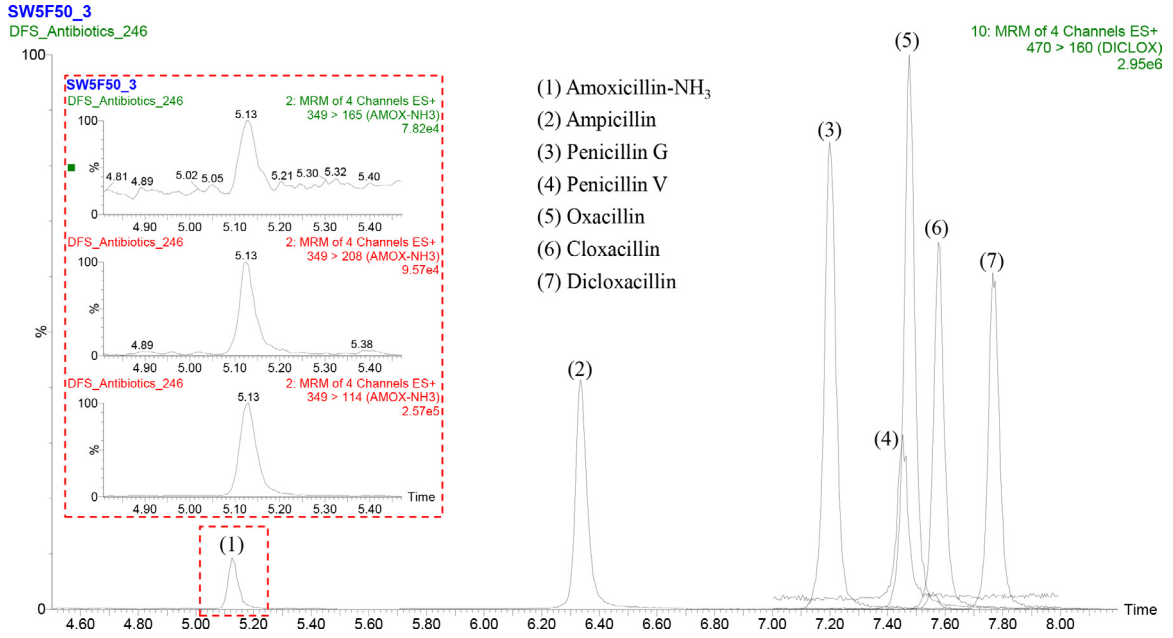

Fig. 5. Chromatographic separation of penicillins studied in sample SW5 spiked at 50 ng L^{-1}. SRM transitions for amoxicillin-NH_3 are also included.

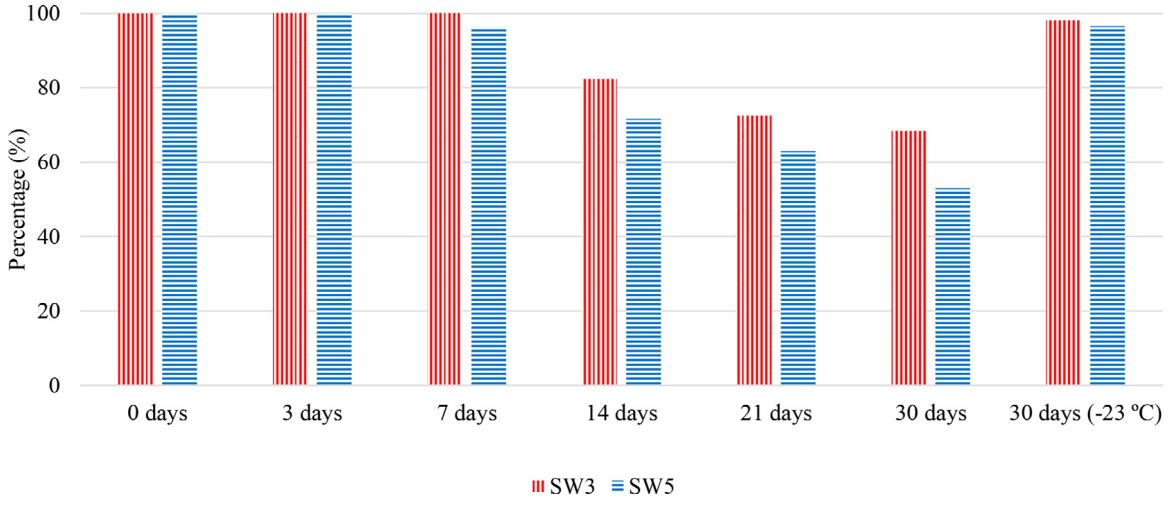

Fig. 6. Stability of amoxicillin in two different surface water samples at 4 °C and −23 °C.

[8], no penicillins were detected. Finally, the analysis of effluent wastewater samples revealed the presence of cloxacillin in three of the four samples collected from the Castelló WWTP, always at concentration levels below LOQ (50 ng L^{-1}) (see an example in **Figure S5**).

4. Conclusions

In this work, the analytical challenges associated to the determination of amoxicillin and related penicillins in water, in the light of the requirements of the European Watch List, have been addressed. A UHPLC-MS/MS methodology based on direct injection has been optimized using a reversed-phase column that allows working with 100% aqueous mobile phase, and reaching an objective LOQ of 50 ng L^{-1} (method satisfactorily validated at this level) and LODs below 10 ng L^{-1}. The direct injection of aqueous samples allowed a substantial reduction in time, and in the use of consumables, such as SPE cartridges, solvents or filters, without compromising method performance, facilitating in this way the application of green chemistry for water pollution monitoring

The use of amoxicillin-ILIS allowed an efficient matrix effect correction leading to satisfactory recoveries in all surface water samples tested. For the remaining penicillins, in absence of their own analyte ILIS, QC samples were analysed in order to estimate correction factors (Cf) as a function of the QC recoveries for each sample analysed. The Cf estimated could be easily applied to the samples analysed to obtain more accurate quantitative data.

The analysis of different water samples revealed the presence of ampicillin in surface river water at 116 ng L^{-1}, as well as the detection of cloxacillin in an effluent wastewater sample.

Declaration of Competing Interest

The authors declare that they do not have competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

David Fabregat-Safont: Resources, Investigation, Formal analysis, Visualization, Writing – original draft. **Elena Pitarch:** Supervision, Visualization, Writing – review & editing. **Lubertus Bijlsma:** Formal analysis, Resources, Writing – review & editing. **Ionut Matei:** Investigation, Formal analysis. **Félix Hernández:** Supervision, Funding acquisition, Writing – review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2021.462605.

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